Environmental Health Perspectives Supplements
Vol. 101 (Suppl. 3): 231–236 (1993)

A Perspective on Nonmutagenic Mechanisms in Carcinogenesis

by Raymond W. Tennant

Although there is compelling evidence for multiple mutagenic events in the induction of cancers, there is also substantial evidence in support of nonmutagenic mechanisms. It is proposed that the genetic basis of noninduced or spontaneous tumors, as well as cancers induced by nonmutagens, involves heritable changes in the regulation of gene expression.

Introduction

Cancer is a disease of environment and genetics. There is a strong scientific consensus, codified by the International Agency for Research on Cancer [IARC (*1*)], that environmental factors such as smoking, exposure to sunlight, exposure to certain organic chemicals, and other occupational and environmental factors establish a solid basis for an environmental component in the induction of cancers. Data derived from epidemiological studies have identified differences in the prevalence and types of certain tumors between geographical areas. Also, differences in the rate or frequency of the development of specific cancers in migrant populations provides support for an environmental component (*2,3*).

Likewise, there is compelling evidence for a genetic basis of human cancer. This includes evidence for heritable susceptibilities between populations of humans, for example, skin cancer among fair skinned Anglo-Saxons and evidence for genetic mechanisms in induced carcinogenesis. The role of genetic mechanisms in carcinogenesis was first proposed by Theodore Boveri in 1924 (*4*), who articulated the earliest version of the somatic mutation hypothesis. Subsequently, other evidence for the role of mutagenic changes in carcinogenesis have come from a variety of sources. These lines of evidence include the chromosomal alterations that have been identified in many rodent and human tumors. In fact, most human and rodent tumors that have been examined show generalized chromosomal damage as well as specific chromosomal mutations or translocations (*5*). Also, over the past decade, evidence has emerged associating up to a hundred different dominant genes (i.e., oncogenes) with carcinogenesis. The role of mutations in the activation of these genes has provided a genetic basis for both initiating and promoting events in tumor development (*6*). Subsequently, evidence has emerged for anti-oncogenes or tumor-suppressor genes, which have an important regulatory role in controlling the expression or function of oncogenes (*7*). The function of these suppressor genes can be lost through mutation or translocation. Subsequent observations have provided a scheme in which multiple genetic changes can be identified and associated with sequential alterations giving rise to tumors in humans (*8*).

These observations represent a very brief and only a partial list of the data that are available to support a role for specific genes in carcinogenesis and for specific alterations or mutations in those genes that initiate or promote the carcinogenic process. In the face of such compelling data, it is difficult to consider the possibility that there may be alternative mechanisms of carcinogenesis.

Evidence Supporting Nonmutagenic Mechanisms

One of the strongest lines of evidence in support of nonmutagenic mechanisms are the chemicals that demonstrate no consistent mutagenic properties, yet have the capacity to induce tumors in rodent bioassays (*9*). Our operational definition of a nonmutagen is a chemical or substance that does not demonstrate evidence of one of the structural alerts associated with electrophilic potential and that the chemical does not induce mutations in the Salmonella assay nor induce chromosomal effects when measured *in vivo* (either induction of chromosome aberrations or micronuclei). Although other mechanisms of genotoxicity or mutagenesis exist, for example, interference with chromosomal metabolism or the mechanics of chromosome segregation, there have been no assays identified yet that are capable of resolving those specific properties that appear to be associated with carcinogenesis. The

National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

operational definition that we use, therefore, may miss some proportion of incipient or indirect mutagens, but it provides the highest degree of specificity for carcinogen identification. We believe that this operational definition defines the chemical groups with the highest probability of direct interaction with and damage to DNA. Chemicals that lack these properties comprise a very structurally diverse group (Table 1).

Table 1. Nonmutagenic carcinogens.[a]

Chemical
Aldrin
Allyl isovalerate
11-Aminoundecanoic acid
Benzaldehyde
Benzene
Benzofuran
Benzyl acetate
Butyl benzyl phthalate
C.I. Vat yellow 4
Chlordane (technical grade)
Chlorendic acid
Chlorinated paraffins: C_{12}, 60% chlorine
Chlorinated paraffins: C_{23}, 43% chlorine
Chlorobenzilate
Chlorothalonil
Cinnamyl anthranilate
Decabromodiphenyl oxide
Di(2-ethylhexyl) adipate
Di(2-ethylhexyl) phthalate
1,4-Dichlorobenzene (*p*-dichlorobenzene)
p,p'-Dichlorodiphenyldichloroethylene
Dicofol
N,N'-Diethylthiourea
1,4-Dioxane
Furfural
Furosemide
Heptachlor
Hexachloroethane
Hydroquinone
Isophorone
d-Limonene
Malonaldehyde, sodium salt
Melamine
Mercaptobenzothiazole
α-Methylbenzyl alcohol
Monuron
Nalidixic acid
Nitrilotriacetic acid (NTA)
N-Nitrosodiphenylamine
Pentachloroethane
Pentachlorophenol
Phenylbutazone
Piperonyl sulfoxide
Polybrominated biphenyl mixture (Firemaster FF-1)
Reserpine
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
1,1,1,2,-Tetrachloroethane
1,1,2,2-Tetrachloroethane
Tetrachloroethylene
1,1,2-Trichloroethane
Trichloroethylene (without epichlorohydrin)
2,4,6-Trichlorophenol
Trimethylthiourea
Tris(2-ethylhexyl)phosphate
Zearalenone

[a]All chemicals listed are negative for structural alerts and were negative in Salmonella.

Genetic Basis for Spontaneous Tumors

Another line of evidence that supports a nonmutagenic origin of some cancers can be derived from the occurrence of spontaneous tumors. Virtually all mammalian species have demonstrated evidence of tumors when older individuals have been examined. The best data on the incidence and patterns of spontaneous tumors are derived from studies with inbred mouse and rat strains. Among the most complete data available are those derived from the 2 year rodent bioassays conducted by the National Toxicology Program [NTP (*10,11*)]. In the protocol used to assess carcinogenicity in rodents, there are concurrent controls of 50 mice or rats of each sex that are held for a 104-week exposure period and subsequently undergo complete postmortem evaluation. The thousands of animals that have been studied have demonstrated fairly consistent patterns of spontaneous tumor development, which has been maintained over many generations. Both mice (i.e., $B6C3F_1$ hybrid) and rats (i.e., F344 strain) are housed under highly controlled conditions, and the diets they are fed are well characterized and contain known, but extremely negligible, amounts of potentially carcinogenic substances. The highly defined and controlled environment provides few, if any, sources of carcinogens. Thus, the constancy of the pattern of tumor development within each sex and species indicates that there are particular genetic determinants that are responsible for the occurrence of spontaneous tumors.

The actual frequency of tumors developed at certain sites do fluctuate and over time; for example, the incidence of mammary tumors or leukemia in rats has tended to increase. The increase in these latter tumors has been associated with improvement in maintenance conditions, that is, a reduction in endogenous viruses and bacteria that could decrease the health of the animals and also attributed to improved dietary conditions that result in relatively high weight gain (*11*). Thus, there are environmental factors that can influence the incidence of spontaneous tumors but do not significantly influence the pattern with which these tumors develop. The origin of spontaneous tumors is unclear. A specific genetic influence has identified the high frequency of liver tumors occurring in $B6C3F_1$ mice that is attributed to a locus called *Hcs* (*12*). The genetic basis of other types of spontaneous tumors has not been well studied, but crosses between strains showing high tissue-specific tumor incidence and other strains showing low tumor incidence at the same site, generally results in an intermediate level of tumor expression in the F_1 progeny, suggesting that in most cases the expression of spontaneous tumors is dominant or semidominant (*13*).

Among various ideas proposed to account for spontaneous tumorigenesis is the concept of DNA damage of endogenous origin. That is, mutations that could occur either as a consequence of mistakes in DNA repair and replication mechanisms (*14*) or from damage that occurs as a consequence of normal metabolism through which

various radicals of oxygen, such as superoxide or hydroxyl, are generated (*15–17*). However, it is difficult to reconcile these hypotheses with the spontaneous tumor patterns that develop in the $B6C3F_1$ mice and F344 rats because neither can account for the tissue specificity of spontaneous tumor incidence. If generalized DNA replication or repair errors or oxidative damage were responsible for the spontaneous tumors, one would expect a more generalized pattern of spontaneous tumor development that would be related to either the tissues with the highest level of endogenous cellular proliferation or to those tissues that have the highest levels of endogenous oxidative metabolism. The pattern of tumors observed do not reflect these patterns (*10*), and other mechanisms by which such tumors could arise should be considered.

It also has been proposed that tissues in which spontaneous tumors arise undergo a higher level of spontaneous initiation and that the action of nonmutagenic carcinogens may involve only the promotion or clonal expansion of such spontaneously initiated cells. To evaluate this hypothesis, we have looked at the sites of tumorigenesis that have been associated with exposure to 154 chemicals identified as carcinogens in the NTP bioassays (*9*). As shown in Figure 1, the carcinogens have induced tumors in about 30 tissue sites, but the majority of chemicals can be found to be active at approximately 10 different sites. These frequencies of induced tumorigenesis were then compared to the sites of spontaneous tumorigenesis demonstrated in control animals. One example, male Fischer rats (Fig. 2), shows a high level of spontaneous tumorigenesis in the hematopoietic system, which is a site of relatively high cell proliferation. However, the hematopoietic system is comparable to the adrenal gland in both spontaneous and induced tumor incidence, but the level of endogenous cellular proliferation is significantly lower in adrenal tissue. Another site of relatively high endogenous cell proliferation is the skin, which shows significantly less spontaneous tumorigenesis and was not a site of tumors induced by nonmutagenic carcinogens. Sites of the highest levels of induced tumors were the liver and kidney, which differ considerably in both levels of endogenous oxidative metabolism and spontaneous tumor incidence.

These results, therefore, suggest the following: *a*) not all sites in the body are equally at risk for chemical-induced tumorigenesis, *b*) the sites of induced tumors are not directly related to the level of endogenous cellular proliferation, *c*) the spontaneous patterns of tumorigenesis do not appear to dramatically influence the sites of tumor induction by exogenous chemicals and, *d*) the genetic influence on spontaneous tumors appears to be the predominant factor in their expression. If indirect mutations of oncogenes are involved in the development of spontaneous tumors, the source of those spontaneous mutations is also unclear, and relatively little information is available. The only extensive studies that have been conducted involve the liver in the $B6C3F_1$ mouse. Spontaneous mutations involving the 12th or 13th codon of the v-H-*ras* gene have been identified in many spontaneous and induced tumors (*12*), but mutated or translocated forms of other oncogenes have not been studied as extensively.

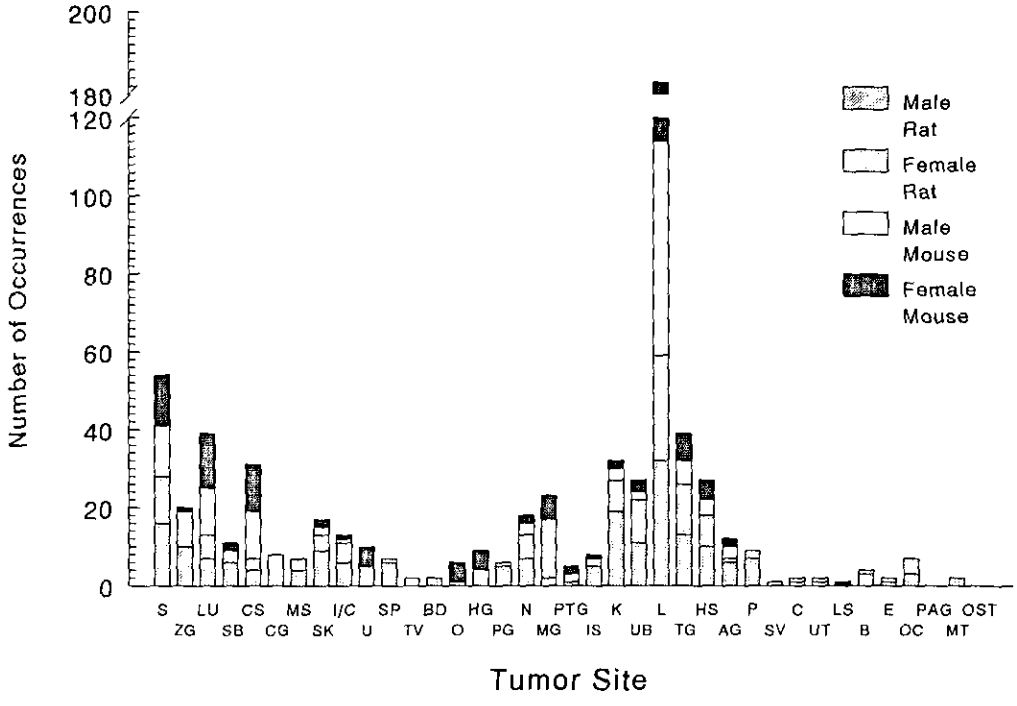

FIGURE 1. Occurrence of tumor sites induced by 154 carcinogens tested by the NTP according to sex and species. See Ashby and Tennant (9) for key to sites.

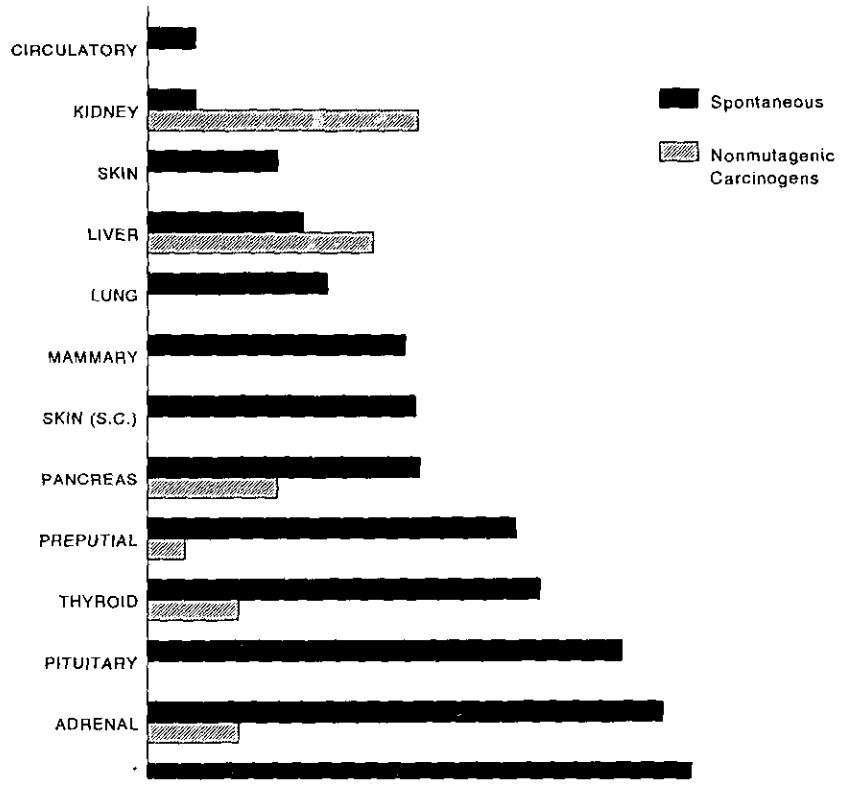

FIGURE 2. The frequencies of induced tumorigenesis by nonmutagenic carcinogens compared to the spontaneous rates for control animals.

Mechanisms of Nonmutagenic Carcinogenesis

The theories proposed to account for the carcinogenicity of nonmutagenic chemicals can be combined into two major groups: indirect mutagenesis and altered gene expression. They are not mutually exclusive mechanisms. It is very possible that some chemicals involve a combination of the two mechanisms, and there are even data to suggest that among the mutagenic carcinogens, indirect mutagenesis or alterations in the expression of important target genes can be critical components in the carcinogenic processes (*19*).

Indirect Mutagenesis

Currently, the most intense speculation about nonmutagenic carcinogens concerns the relationship between toxicity, sustained tissue damage, and induced cellular proliferation or mitogenesis (*20*). Cell proliferation generally refers to compensatory or reparative cell division that is a consequence of toxicity, whereas mitogenesis generally refers to the capacity of a chemical or substance to directly elicit cell division. Investigations by Totter (*15*), Ceruti (*16*), and Ames and Gold (*17*) have proposed that byproducts of the normal oxidative metabolism of cells gives rise to relatively high levels of free radicals such as superoxide or hydroxyl that have the capacity to damage DNA and to induce mutations. Ames and Gold (*17*) have focused on chemicals that induce toxicity and suggest that reparative processes associated with toxic injury such as the infiltration of macrophages can increase the level of oxyradicals. They propose that toxic injury sustains cell proliferation and can promote the development of tumors by providing for the clonal expansion of cells damaged by oxyradicals. Evidence in support of this mechanism has been offered by the identification of 8-hydroxyguanosine. This is an altered DNA base that occurs as a result of oxyradical-induced DNA damage. However, there is no way to determine whether such altered bases occur in healthy cells that are dividing and have the capacity to repair such damage or whether they occur predominantly in cells that are irreversibly injured by toxicity and thus could not contribute to either the proliferative process or to subsequent development of tumors.

A second mechanism by which endogenous sources of DNA damage could arise has been summarized by Loeb (*14*). He has proposed that lesions induced in DNA by mistakes in replication and repair processes induce spontaneous mutations and that such mutational events could account for a significant proportion of endogenously initiated cells.

The major argument against the amplification or clonal expansion of endogenous DNA damaged cells or mutated cells (the "mitogenesis and mutagenesis" theory) come from two lines of evidence. The first is derived from an extensive evaluation of a number of chemicals that induce organ-specific toxicity in the 2-year rodent bioassays.

Toxicity that is sustained for much of the 104-week period of chemical exposure in these bioassays can result in proliferative responses such as induction of hyperplasia in specific tissues. Such changes can occur in the absence of neoplasia (*19*). The bioassays are carried out for 2 years, which represents approximately 60% of the life span of the animal. The animals undergo complete postmortem examination at the end of the bioassay period, and there is little basis for arguing that tumors might be detected if the injury was sustained longer or if the animals were observed longer.

The second line of evidence is based on the pattern of spontaneous tumors that occurs in the mice and rats used in the bioassay. As discussed previously, the data do not support generalized induction of spontaneously initiated cells either by errors in proliferation and repair or by oxidative damage. Comparisons of the sites of spontaneous tumors with the sites of highest levels of endogenous cellular proliferation or of oxidative metabolism show very little relationship to the pattern of spontaneous tumors. Therefore, it appears unlikely that the spontaneously occurring tumors in rodents can be directly accounted for by indirect mutagenesis mechanisms. These data do support the concept that the spontaneous tumors arise as a result of specific genetic determinants in the animal. This does not exclude the possibility that some chemicals may have the capacity to induce indirect oxidative damage or to increase the level of cellular and repair proliferation mistakes, and to subsequently clonally amplify these mistakes when they occur in an oncogene or suppressor gene. It is a plausible mechanism for some tumorigenesis, but it is unlikely to account for the full range of carcinogenic effects observed among the large number of nonmutagenic carcinogens.

Altered Gene Expression

If the clonal amplification of spontaneous or indirectly initiated cells is not a common mechanism of carcinogenesis, then how may proliferative processes give rise to tumors? There are different lines of evidence that can be interpreted to support a role for alterations in the expression of one or more of the critical proto-oncogenes or proto-suppressor genes, that is, the endogenous forms of the oncogenes or suppressor genes play critical roles in the complex regulatory pathways that control normal cell functions. An analogy can be drawn from the processes of differentiation wherein sequential changes in the expression of genes and the responses of differentiating cells to the gene products result in heritable alterations in the pattern of gene expression. Such changes are acquired by, and often modified in, progeny cells. When appropriate stages of differentiation are reached, the pattern of gene expression can become fixed and subsequently inherited by daughter cells arising in those tissues. Therefore, it is possible that some chemicals or environmental agents can act by altering the expression of these critical regulatory genes and give rise to progeny cells in which the heritable phenotypic change provides a growth advantage. The development of tumors in response to so-called "solid-state" carcinogens, such as plastic strips, films, or calculi could involve such a mechanism. The cells adapt to growth in the presence of, or on the foreign objects. Progressively, more dysregulated cells can emerge with a growth advantage that eventually becomes a tumor phenotype (*21*). The question of whether mutations are induced in critical targets in such cells has not been addressed. Therefore, it is inadequate to assume that mutations are required to elicit neoplastic growth under these conditions.

Hormonal carcinogenesis provides a second line of evidence. The profound changes on normal regulatory processes induced by hormones involve complex interactions with surface or intracellular receptors and the transduction of signals to the nucleus where changes in gene expression are affected by the action of various transcription factors. Oncogenes that play roles in these processes have been identified by virtue of the mutated forms of the genes that exist in and were transduced by retroviruses. However, there are normal cellular counterparts for such genes. For example, the *erb*A protein functions as an intracellular receptor for thyroid hormone (T_3) and functions as a negative regulator of transcription (*22*). The mutated form of *erb*A has been demonstrated to play a role in tumorigenic processes. However, constitutive expression of the endogenous c-*erb*A proto-oncogene can plausibly result in similar events. For example, if a chemical can function as a ligand for the thyroid hormone receptor and significant levels of the chemical are present for protracted periods of time, it is possible that the normal regulatory functions of the receptor will be subverted and that the *erb*A gene product would be constitutively produced. The consequence could be a dysregulated pattern of cellular proliferation because there is selection for more rapidly proliferating cell populations. Daughter cells also required to exist in the presence of the chemical would also possess the altered phenotype. Such dysregulated proliferating cells would provide a fertile environment in which subsequent genetic changes could occur and lead to a malignant phenotype. Thus, the proliferation of thyroid cells may be fundamentally different in the presence of a nonmutagenic chemical that can alter the process of gene expression.

Pathways such as that proposed for the *erb*A oncogene provide a basis for viewing the emergence of some cancers as an adaptive process. In this hypothesis it is not the direct action of the chemical that induces specific changes in cells, but rather that the chemical elicits adaptive responses on the part of cells that lead to deregulated growth patterns and the emergence of neoplastic variants (*21*). While this may seem to be a minor distinction between the actions of some nonmutagenic chemicals, it has important implications. For example, the adaptive process may be intrinsically more reversible than the inductive process. In the absence of the chemical, reversion to a normal phenotype may be possible. Numerous examples of reversion or remodeling have been seen in studies of hepatocarcinogenesis and in the neoplastic transformation of cells in culture (*21*). However, mutation is a process and mutants

are a product that can be studied and quantitated far more readily than can changes in the patterns of gene transcription. It is necessary to explore further the complex molecular interactions of transcription factors with DNA binding sites and to determine if specific chemicals can dysregulate the expression of critical control genes in ways that do not involve changes in DNA sequence (*23*).

Thus, I am proposing that at least some portion of cancers are diseases of transcription that arise through mistakes in the complex process of transcriptional regulation and that some chemicals have the capacity to illicit such changes independent of their ability to stimulate cellular proliferation. Induced cellular proliferation, therefore, may be an essential component allowing for the clonal amplification of transcriptionally altered cells. However, chemicals that can induce cellular proliferation directly through a mitogenic effect, or indirectly through eliciting a compensatory response to the toxic effects of the chemical, are not necessarily carcinogenic. Other properties of the chemical, related to their ability to specifically interfere with the transcriptional process, may be the important property that distinguishes this class of nonmutagenic carcinogen from both other nonmutagens and mutagens that are carcinogens.

REFERENCES

1. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans. Overall Evaluations of Carcinogenicity, Supplement 7. International Agency for Research on Cancer, Lyon, 1987.
2. Cairns, J. The origin of human cancers. Nature 289: 353–357 (1981).
3. Henderson, B. E., Ross, R. K., and Pike, M. C. Toward the primary prevention of cancer. Science 254: 1131–1137 (1991).
4. Boveri, T. The Origin of Malignant Tumors. Williams and Wilkins, Baltimore, MD, 1929.
5. Solomon, E., Borrow, J., and Goddard, A. D. Chromosome aberrations and cancer. Science 254: 1153–1160 (1991).
6. Bishop, M. Molecular themes in oncogenesis. Cell 64: 235–248 (1991).
7. Weinberg, R. A. Tumor suppressor genes. Science 254: 1138–1145 (1991).
8. Fearon, E. R., and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell 61: 759–767 (1990).
9. Ashby, J., and Tennant, R. W. Definitive relationships between chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the US NTP. Mutat. Res. 257: 229–306 (1991).
10. Haseman, J. K., Huff, J. E., Rao, G. N., Arnold, J. E., Boorman, G. A., and McConnell, E. E. Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57B1/6N × C3H/Hen) F1 (B6C3F1) mice. J. Natl. Cancer Inst. 75: 975–984 (1985).
11. Haseman, J. K., and Rao, G. N. Effects of corn oil, time-related changes, and inter-laboratory variability on tumor occurrence in control Fischer 344 (F344/N) rats. Toxicol. Pathol. 20: 52–60 (1992).
12. Drinkwater, N. R., and Ginsler, J. J. Genetic control of hepatocarcinogenesis in C57B1/6J and C3H/Hev inbred mice. Carcinogenesis 7: 1701–1707 (1986).
13. Eker, R., and Mossige, J. A. A dominant gene for renal adenomas in the rat. Nature 189: 858–859 (1961).
14. Loeb, L. A. Endogenous carcinogenesis: molecular oncology into the twenty-first century-presendial address. Cancer Res. 49: 5489–5496 (1989).
15. Totter, J. Spontaneous cancer and its possible relationship to oxygen metabolism. Proc. Natl. Acad. Sci. USA 77: 1763–1767 (1980).
16. Cerutti, P., Larsson, R., and Krupitza, G. Mechanisms of Oxidadant Carcinogenesis in Genetic Mechanisms in Carcinogens and Tumor Progression. Wiley-Liss, Inc., 1990, pp. 69–82.
17. Ames, B. N., and Gold, L. S. Too many rodent carcinogens: mitogenesis increases mutagenesis. Science 249: 970–971 (1990).
18. Reynolds, S. H., Stowers, S. J., Patterson, R. M., Maronpot, R. M., Aaronson, S. A., Anderson, M. W. Activated oncogenes in B6C3F1 mouse liver tumors: implications for risk assessment. Science 237: 1309–1316 (1987).
19. Tennant, R. W., Elwell, M. R., Spalding, J. W., and Griesemer, R. A. Evidence that toxic injury is not always associated with induction of chemical carcinogenesis. Mol. Carcinog. 4: 420–440 (1991).
20. Cohen, S. M., and Ellwein, L. B. Genetic errors, cell proliferation and carcinogenesis. Cancer Res. 51: 6493–6505 (1991).
21. Farber, E., and Rubin, H. Cellular adaptation in the origin and development of cancer. Cancer Res. 51: 2751–2761 (1991).
22. Damm, K., Thompson, C. C., and Evans, R. M. Protein encoded by v-erb A functions as a thyroid-hormone receptor antagonist. Nature 339: 593–597 (1989).
23. Issemann, I., and Green, S. Activation of a member of the steriod hormone receptor superfamily by peroxisome proliferators. Nature 347: 645–650 (1990).